

(19)



Europäisches Patentamt  
European Patent Office  
Office européen des brevets

(11) Publication number:

**0 176 321**  
**A1**

(12)

## EUROPEAN PATENT APPLICATION

(21) Application number: 85306667.8

(22) Date of filing: 19.09.85

(51) Int. Cl.<sup>4</sup>: **C 12 N 15/00**  
**C 12 N 5/00, C 12 N 1/20**  
**//C12R1:19, C12R1:465,**  
**C12R1:485, C12R1:54**

(30) Priority: 27.09.84 US 655178  
07.06.85 US 742172

(43) Date of publication of application:  
02.04.86 Bulletin 86/14

(84) Designated Contracting States:  
BE CH DE FR GB IT LI NL SE

(71) Applicant: **ELI LILLY AND COMPANY**  
**Lilly Corporate Center**  
**Indianapolis Indiana 46285(US)**

(72) Inventor: **Rao, Ramachandra Nagaraja**  
**6818 Hoover Road**  
**Indianapolis Indiana 46260(US)**

(72) Inventor: **Stanzak, Richard Kale**  
**Route 2 Box 286-c**  
**Poland Indiana 47868(US)**

(74) Representative: **Hudson, Christopher Mark et al,**  
**Erl Wood Manor**  
**Windlesham Surrey GU20 6PH(GB)**

(54) **Recombinant dna cosmid shuttle vectors.**

(57) Novel recombinant DNA cosmid shuttle vectors, transformed host cells, and a method of using the cosmid vectors in the construction of genomic DNA libraries are described. The vectors demonstrate the incorporation of both the size selection and *in vitro* packaging mechanisms of lambda into a *Streptomyces-E. coli* shuttle vector.

EP 0 176 321 A1

**BEST AVAILABLE COPY**

## RECOMBINANT DNA COSMID SHUTTLE VECTORS

The present invention provides novel recombi-  
5 nant DNA cosmid shuttle vectors comprising replicons  
that are functional in Escherichia coli and Strepto-  
myces, a DNA segment that contains two or more cos  
elements of bacteriophage lambda and one or more DNA  
segments that convey resistance to antibiotics. The  
10 invention further comprises transformants of these  
vectors. A method of using the cosmid shuttle vectors  
to construct genomic DNA libraries also is disclosed.

Cosmids are vectors specifically designed for  
cloning large fragments of foreign DNA. These vectors  
15 are modified plasmids which contain a plasmid replicon,  
a selectable drug resistance marker and the lambda cos  
site. Due to their relatively small size and the  
presence of the lambda cos element, cosmids can accept  
inserts of up to 30-45 kilobases (kb) and utilize the  
20 lambda in vitro packaging system to positively select  
for large size inserts. These vectors, therefore,  
provide an efficient mechanism to introduce foreign DNA  
into bacterial cells.

The present invention provides antibiotic  
25 resistance-conferring bifunctional cosmid vectors for  
use in E. coli and Streptomyces host cells. Bifunc-  
tional constructions are particularly advantageous  
because amplification and manipulation of vectors can be  
performed faster and more conveniently in E. coli than

in Streptomyces. Thus, after desired recombinant DNA procedures are accomplished within the E. coli host system, the particular plasmid DNA can be removed and then transformed into a Streptomyces host cell. It may  
5 also be possible to transfer directly the plasmid DNA to a Streptomyces host cell by means of a cell-to-cell fusion or phage particle-to-cell fusion. Gene cloning and expression of products in Streptomyces are highly desirable because the organism is substantially non-  
10 pathogenic and ordinarily does not produce endotoxins. Heretofore, the development and exploitation of recombinant DNA technology in the above organisms has been retarded and time-consuming because of the general lack of efficient cloning systems available to accomodate  
15 large segments of DNA. The vectors of the present invention can accomodate large inserts of DNA, are functional as well as selectable in both Streptomyces and E. coli host strains and, therefore, represent a significant advance in the technical art.

20 The present invention further provides cosmid cloning vectors which contain multiple lambda cos sites. The presence of multiple cos sites on a single vector eliminates the need to prepare separate cosmid arms. Consequently, the construction of genomic libraries is  
25 facilitated by the structural composition of the present vectors. The present invention also provides a convenient method to construct genomic DNA libraries using the cosmid shuttle vectors provided. Presently, there are three systems available for constructing a genomic  
30 DNA library: transformation of a bacterial cell by

plasmid DNA (L. Clark and J. Carbon, 1976, Cell 9:91);  
transduction of a bacterial cell by lambda bacteriophage  
vectors (Lawn et al., 1978, Cell 15:1157); and trans-  
duction of a bacterial cell by cosmid vectors (Collins  
5 et al., 1978, Proc. Natl. Acad. Sci., 75:4242). However,  
lambda vectors are limited in their ability to accomodate DNA  
inserts of up to 20 kb. and large plasmids have low  
transformation efficiencys. Thus, the use of cosmid  
vectors to clone large DNA fragments is preferred over  
10 both plasmid and lambda bacteriophage vectors. There is a  
twofold advantage of using cosmid vectors to construct  
genomic libraries over plasmid or lambda bacteriophage  
vectors. First, the ability of a cosmid vector to  
accomodate large DNA inserts preserves the original  
15 linkage relationships of the inserted genome. This  
preservation is, however, dependent on the details of  
the cloning procedure and is independent of the cloning  
system. Since larger inserts cover the entire genome,  
statistically, in a limited number of colonies, the  
20 screening process is thereby reduced. Secondly, the  
relative ease of preparation of DNA confers a beneficial  
advantage to the present cosmid vectors.

The specific advantage of the method according  
to the invention over known cosmid vectors is the  
25 ability of the present vectors to be grown and amplified  
in E. coli and then shuttled into Streptomyces host  
cells for subsequent functional analysis of the cloned  
DNA. The vectors are relatively small, versatile and  
can be transformed and selected in any Streptomyces cell  
30 that is sensitive to an antibiotic for which resistance

X-6422A

-4-

is conveyed and wherein the Streptomyces plasmid origin of replication is functional. Because more than seventy percent of naturally occurring antibiotics are produced by Streptomyces strains, it is desirable to develop  
5 cloning systems and vectors that are applicable to this industrially important group. The present invention provides such vectors and thus allows for the shuttling and cloning of genes into Streptomyces both for increasing the yields of known antibiotics as well as for the  
10 production of new antibiotics and antibiotic derivatives.

The present invention provides vehicles for cloning DNA into Streptomyces host cells and also allows for the convenient selection of transformants. Since transformation is a very low frequency event, such a  
15 functional test is a practical necessity for determining which cell(s), of among billions of cells, has acquired the plasmid DNA. This is important because the foreign DNA sequences that are themselves non-selectable can be inserted into the vectors and, upon transformation,  
20 cells containing the vector and the particular DNA sequence of interest can be isolated by appropriate phenotypic selection.

For purposes of the present invention as disclosed and claimed, the following terms are defined:  
25 Recombinant DNA Cloning Vector - any autonomously replicating agent, including but not limited to plasmids, comprising a DNA molecule to which one or more additional DNA segments can or have been added.

Cosmid - a plasmid carrying the ligated cohesive ends  
30 (cos) of bacteriophage; as a result, the plasmid DNA can

be packaged either in vitro in the phage coat or in vivo using suitable E. coli strains.

5     cos sequence - a cohesive end site comprising a 12 nucleotide sequence from bacteriophage lambda that is recognized by the lambda-specific packaging proteins.

Library - a collection of cloned fragments of DNA, which together represent an entire genome.

10    Restriction Fragment - any linear DNA generated by the action of one or more restriction enzymes.

Sensitive Host Cell - a host cell that cannot grow in the presence of a given antibiotic without a DNA segment that confers resistance thereto.

15    Transformation - the introduction of DNA into a recipient host cell that changes the genotype and consequently results in a change in the recipient cell.

Transformant - a recipient host cell that has undergone transformation.

20    E. coli Replicon - a DNA sequence that controls and allows for replication of a plasmid or other vector in E. coli.

Streptomyces Replicon - a DNA sequence that controls and allows for replication of a plasmid or other vector in Streptomyces.

25    Ap<sup>R</sup> - the ampicillin resistant phenotype.

Am<sup>R</sup> - the apramycin resistant phenotype.

Tsr<sup>R</sup> - the thiostrepton resistant phenotype.

Nm<sup>R</sup> - the neomycin resistant phenotype.

X-6422A

-6-

The present invention provides a recombinant DNA cosmid shuttle vectors comprising:

- a) a replicon that is functional in E. coli,
- b) a replicon that is functional in  
5        Streptomyces,
- c) a DNA segment that contains two or more  
      cos sequences of bacteriophage lambda,  
      and
- d) one or more DNA segments that convey  
10       resistance to at least one antibiotic  
      when transformed into a sensitive re-  
      strictionless host cell.

The invention further comprises transformants of the aforementioned vectors.

15       Vectors of the present invention represent novel hybrids between a Streptomyces vector and a cosmid. For example, cosmid vector pKC420 can replicate autonomously in Streptomyces and in E. coli since it contains replicons from both organisms. In addition, selectable  
20       markers are present for both organisms ( $Ap^R$  in E. coli and  $Am^R$  in both E. coli and Streptomyces) providing a convenient means to select transformants. Furthermore, the bacteriophage lambda cos sequences allow the new  
25       vector to be packaged in vitro and transformed into E. coli. The recombinant plasmids then can be used to transform Streptomyces host cells. Thus, given the presence of  $\lambda$  cos sequences in the present shuttle vectors, the cloning advantages inherent to cosmid vectors are now applicable to Streptomyces.

Cosmid shuttle vector pKC420 is approximately 10.6 kb and contains several restriction sites which are particularly advantageous for molecular cloning. Cosmid pKC420 can be isolated conventionally from E. coli K12 DH1/pKC420, a constructed strain deposited and made part of the stock culture collection of the Northern Regional Research Laboratory, Peoria, Illinois 61604. It is available to the public as a source and stock reservoir of the cosmid under the accession number NRRL B-15837.

A detailed restriction site and functional map of cosmid pKC420 is presented in Fig. 1 of the accompanying drawings. For purposes of the present application, Fig. 1 and all subsequent figures are not drawn to scale.

Cosmid pKC420, useful directly as a cloning vector, also can be used to construct derivative vectors within the scope of the present invention. Cosmid pKC420 can be restricted and ligated to one or more antibiotic resistance conferring DNA fragments, exemplified herein for illustrative purposes by the thio-strepton resistance conferring ~1 kb BclI restriction fragment of plasmid pIJ702 (ATCC 39155), the Tn903 neomycin resistance conferring ~1.5 kb EcoRI restriction fragment of plasmid pUC4K (NRRL B-15836), and the Tn5 neomycin resistance conferring ~1.5 kb HindIII-SalI restriction fragment of plasmid pKC7 (ATCC 37084), to form vectors illustrative of the present invention. Plasmids pIJ702 and pKC7 can be isolated from strains deposited and made part of the stock culture collection of the American Type Culture Collection, Rockville,



X-6422A

-8-

Maryland 20852, and are available to the public as a source and stock reservoir of their plasmids under the accession numbers ATCC 39155 and 37084, respectively. Plasmid pUC4K, the source of the neomycin resistance  
5 conferring fragment, is a strain deposited and made part of the stock culture collection of the Northern Regional Research Laboratory, Peoria, Illinois 61604 and is available to the public as a source and stock reservoir of the plasmid under the accession number NRRL B-15836.

10 For convenience and ease of construction, the thiostrepton resistance conferring ~1 kb BclI fragment is inserted into cosmid pKC420 at the unique BamHI restriction site. The resulting recombinant DNA then is ligated to produce cosmids illustrative of the present  
15 invention. Phenotypically desired recombinant plasmids of two orientations result depending upon the orientation of the inserted DNA fragment. Thus, the insertion of the ~1 kb BclI restriction fragment into cosmid pKC420 results in illustrative cosmids pKC427 and pKC427A.

20 Various cosmid pKC420 restriction sites can be used for the insertion of DNA segments provided that the replicons, selectable markers and other necessary plasmid functions are not disrupted. Those skilled in the art understand or can determine readily which sites  
25 are advantageous for the ligation or insertion of a particular DNA segment.

Although the thiostrepton and neomycin anti-biotic resistance conferring DNA segments are, for illustrative purposes, respectfully exemplified by the  
30 ~1 kb BclI restriction fragment of pIJ702, the ~1.5 kb

EcoRI restriction fragment of plasmid pUC4K, and the ~1.5 kb HindIII-SalI restriction fragment of plasmid pKC7, those skilled in the art can construct and substitute, either individually or in combination, other DNA segments that also confer resistance to the aforementioned antibiotics. Other thiostrepton resistance conferring DNA segments include, for example, the ~1.6 kb BamHI restriction fragment of plasmid pLR2. Other neomycin resistance conferring DNA segments include, for example, the ~3.5 kb PstI restriction fragment and the ~3.4 kb BamHI restriction fragment of plasmid pLR1. Plasmids pLR2 and pLR1 are constructed in accordance with U.S. Patent No. 4,416,994 and the constructions are incorporated herein by reference.

Additional DNA segments conferring resistance to the above or to different antibiotics such as, for example, hygromycin, chloramphenicol, streptomycin, viomycin, tylosin, erythromycin, and the like also can be constructed and used for purposes of the present invention. Moreover, various functional derivatives of the above described antibiotic resistance conferring DNA segments can be constructed by adding, eliminating, or substituting nucleotides in accordance with the genetic code. Those skilled in the art will understand that ligation of these, or any other antibiotic resistance conferring DNA segments, with cosmid pKC420 DNA, results in cosmid shuttle vectors that are within the scope of the present invention.

Cosmid pKC420 and any cosmids derived therefrom, as well as the antibiotic resistance conferring

DNA segments, can be conveniently modified to facilitate subsequent ligation. For example, the addition of an EcoRI molecular linker with EcoRI-BamHI-EcoRI sites to cosmid pKC427 provides for the construction of a specific  
5 restriction site, such as, for example, a BamHI restriction site, that is useful for ligation or for other purposes known in the art. Moreover, the various restriction fragments also can be modified by adding, eliminating, or substituting nucleotides to alter  
10 characteristics and to provide a variety of unique or additional restriction sites. Those skilled in the art understand nucleotide chemistry and the genetic code and thus which nucleotides are interchangeable and which DNA modifications are desirable for a specific purpose.

15 The present vectors are not limited to the use of a specific replicon from an E. coli or Streptomyces plasmid. Although the E. coli functional replicon exemplified in the present cosmid vectors is from plasmid pBR322, other E. coli replicon containing  
20 fragments can be obtained from, for example, plasmids pBR324 and pBR325 (disclosed in Bolivar, F., 1978, Gene 4:121), plasmid pBR328 (disclosed in Soberon, X., 1980, Gene 9:287), or the like, to produce novel bifunctional cosmids. Additionally, other Streptomyces  
25 replicon containing fragments can be substituted for the Streptomyces replicon. These replicon containing fragments include, but are not limited to, replicons from plasmids SCP2 and SCP2\* (disclosed in Bibb and Hopwood, 1981, J. Gen. Microbiol. 126:427), SLP1 (disclosed in Bibb, M.J., 1981, Mol. Gen. Genet. 184:230),  
30

pEL103 (NRRL 12549), pFJ265 (disclosed in Jones, M.D.,  
et al., 1984, Plasmid 11:92) and pHJL210 (NRRL B-15824).  
Those skilled in the art will understand that ligation  
of these, or other E. coli or Streptomyces replicon  
5 containing fragments, results in cosmid shuttle vectors  
that are within the scope of the present invention.

The recombinant DNA cosmid shuttle vectors of  
the present invention are not limited for use in a  
single species or strain of Streptomyces. To the con-  
10 trary, the vectors are broadly applicable and can be  
transformed into host cells of many Streptomyces taxa,  
particularly restrictionless strains of economically  
important taxa that produce antibiotics such as amino-  
glycoside, macrolide,  $\beta$ -lactam, polyether, and glyco-  
15 peptide antibiotics. Such restrictionless strains are  
selected and isolated readily from Streptomyces taxa by  
conventional procedures well known in the art.  
(Lomovskaya et al., 1980, Microbiological Reviews  
44:206). Host cells of restrictionless strains lack  
20 restriction enzymes and, therefore, do not cut or degrade  
plasmid DNA upon transformation. For purposes of the  
present application, host cells containing restriction  
enzymes that do not cut any of the restriction sites of  
the present recombinant cosmids also are considered  
25 restrictionless.

Preferred host cells of restrictionless  
strains of Streptomyces taxa that produce aminoglycoside  
antibiotics and in which the present vectors are espe-  
cially useful and can be transformed, include restric-  
30 tionless cells of, for example: Streptomyces

- kanamyceticus (kanamycins), S. chrestomyceticus (aminosidine), S. griseoflavus (antibiotic MA 1267), S. microsporeus (antibiotic SF-767), S. ribosidificus (antibiotic SF733), S. flavopersicus (spectinomycin),
- 5 S. spectabilis (actinospectacin), S. rimosus forma paromomycinus (paromomycins, catenulin), S. fradiae var. italicus (aminosidine), S. bluensis var. bluensis (bluensomycin), S. catenulae (catenulin), S. olivoreticuli var. cellulophilus (destomycin A), S.
- 10 lavendulae (neomycin), S. albogriseolus (neomycins), S. albus var. metamycinus (metamycin), S. hygrosopicus var. sagamiensis (spectinomycin), S. bikiniensis (streptomycin), S. griseus (streptomycin), S. erythrochromogenes var. narutoensis (streptomycin), S.
- 15 poolensis (streptomycin), S. galbus (streptomycin), S. rameus (streptomycin), S. olivaceus (streptomycin), S. mashuensis (streptomycin), S. hygrosopicus var. limoneus (validamycins), S. rimofaciens (destomycins), S. hygrosopicus forma glebosus (glebomycin), S. fradiae
- 20 (hybrimycins neomycins), S. eurocidicus (antibiotic Al6316-C), S. aquacanus (N-methyl hygromycin B), S. crystallinus (hygromycin A), S. noboritoensis (hygromycin), S. hygrosopicus (hygromycins), S. atrofaciens (hygromycin), S. kasugaspinus (kasugamycins), S. kasugaensis (kasugamycins), S. netropsis
- 25 (antibiotic LL-AM31), S. lividus (lividomycins), S. hofuensis (seldomycin complex), and S. canus (ribosyl paromamine).

- 30 Preferred host cells of restrictionless strains of Streptomyces taxa that produce macrolide

antibiotics and in which the present vectors are especially useful and can be transformed, include restrictionless cells of, for example: Streptomyces caelestis (antibiotic M188), S. platensis (platenomycin), S. rochei var. volubilis (antibiotic T2636), S. venezuelae (methymycins), S. narbonensis (josamycin, narbomycin), S. fungicidicus (antibiotic NA-181), S. griseofaciens (antibiotic PA133A, B), S. roseocitreus (albocycline), S. bruneogriseus (albocycline), S. roseochromogenes (albocycline), S. cinerochromogenes (cineromycin B), S. albus (albo-mycetin), S. felleus (argomycin, picromycin), S. rochei (lankacidin, borrelidin), S. violaceoniger (lankacidin), S. griseus (borrelidin), S. maizeus (ingramycin), S. albus var. coilmyceticus (coleimycin), S. mycarofaciens (acetyl-leukomycin, espinomycin), S. hygrosopicus (turimycin, relomycin, maridomycin, tylosin, carbo-mycin), S. griseospiralis (relomycin), S. lavendulae (aldgamycin), S. rimosus (neutramycin), S. deltae (deltamycins), S. fungicidicus var. espinomyceticus (espinomycins), S. furdicidicus (mydecamycin), S. ambofaciens (foromacidin D), S. eurocidicus (methymycin), S. griseolus (griseomycin), S. flavochromogenes (amaromycin, shincomycins), S. fimbriatus (amaromycin), S. fasciculus (amaromycin), S. erythreus (erythromycins), S. antibioticus (oleandomycin), S. olivochromogenes (oleandomycin), S. spinichromogenes var. suragaoensis (kujimycins), S. kitasatoensis (leucomycin), S. narbonensis var. josamyceticus (leucomycin A3, josamycin), S. albogriseolus (mikonomycin),

X-6422A

-14-

S. bikiniensis (chalcomycin), S. cirratus (cirramycin),  
S. djakartensis (niddamycin), S. eurythermus (angola-  
mycin), S. fradiae (tylosin, lactenocin, macrocin), S.  
goshikiensis (bandamycin), S. griseoflavus (acumycin),  
5 S. halstedii (carbomycin), S. tendae (carbomycin), S.  
macrosporeus (carbomycin), S. thermotolerans (carbo-  
mycin), and S. albireticuli (carbomycin).

Preferred host cells of restrictionless  
strains of Streptomyces taxa that produce  $\beta$ -lactam  
10 antibiotics and in which the present vectors are  
especially useful and can be transformed, include  
restrictionless cells of, for example: Streptomyces  
lipmanii (A16884, MM4550, MM13902), S. clavuligerus  
(A16886B, clavulanic acid), S. lactamdurans (cephamycin  
15 C), S. griseus (cephamycin A, B), S. hygroscopicus  
(deacetoxycephalosporin C), S. wadayamensis (WS-3442-D),  
S. chartreusis (SF 1623), S. heteromorphus and S. pana-  
yensis (C2081X); S. cinnamomensis, S. fimbriatus, S.  
halstedii, S. rochei and S. viridochromogenes (cepha-  
20 mycins A, B); S. cattleya (thienamycin); and S. oliva-  
ceus, S. flavovirens, S. flavus, S. fulvoviridis, S.  
argenteolus, and S. sioyaensis (MM 4550 and MM 13902).

Preferred host cells of restrictionless  
strains of Streptomyces taxa that produce polyether  
25 antibiotics and in which the present vectors are  
especially useful and can be transformed, include  
restrictionless cells of, for example: Streptomyces  
albus (A204, A28695A and B, salinomycin), S. hygro-  
scopicus (A218, emericid, DE3936), A120A, A28695A and  
30 B, etheromycin, dianemycin), S. griseus (grisorixin),

S. conglobatus (ionomycin), S. eurocidicus var. astero-  
cidicus (laidlomycin), S. lasaliensis (lasalocid),  
S. ribosidificus (lonomycin), S. cacaoi var. asoensis  
5 (lysocellin), S. cinnamomensis (monensin), S. aureo-  
faciens (narasin), S. gallinarius (RP 30504), S. long-  
woodensis (lysocellin), S. flaveolus (CP38936), S.  
mutabilis (S-11743a), and S. violaceoniger (nigericin).

Preferred host cells of restrictionless  
strains of Streptomyces taxa that produce glycopeptide  
10 antibiotics and in which the present vectors are  
especially useful and can be transformed, include  
restrictionless cells of, for example: Streptomyces  
orientalis and S. haranomachiensis (vancomycin); S.  
candidus (A-35512, avoparcin), S. eburosporeus (LL-AM  
15 374), and S. toyocaensis (A47934).

Preferred host cells of other Streptomyces  
restrictionless strains in which the present vectors  
are especially useful and can be transformed, include  
restrictionless cells of, for example: Streptomyces  
20 coelicolor, S. granoloruber, S. roseosporus, S.  
lividans, S. tenebrarius, S. acrimycins, S. glaucescens,  
S. parvulin, S. pristinaespiralis, S. violaceoruber, S.  
vinaceus, S. virginiae, S. espinosus, and S. azureus.

In addition to the representative Strepto-  
25 myces host cells described above, the present vectors  
are also useful and can be transformed into cells of  
restrictionless strains of other taxa such as, for  
example: Bacillus, Staphylococcus and related Actino-  
mycetes, including Streptosporangium, Actinoplanes,  
30 Nocardia, and Micromonospora. Thus, the vectors of the



present invention have wide application, are useful in and can be transformed into host cells of a variety of organisms.

While all the embodiments of the present invention are useful, some of the present recombinant DNA cloning vectors and transformants are preferred. Accordingly, preferred vectors are cosmids pKC420, pKC427, pKC428, pKC448, pKC462A, and pKC467; and preferred transformants are Streptomyces ambofaciens/pKC420, S. ambofaciens/pKC427, S. ambofaciens/pKC428, S. ambofaciens/pKC448, S. ambofaciens/pKC462A, S. ambofaciens/pKC467, E. coli K12 SF8/pKC420, E. coli K12 SF8/pKC427, E. coli K12 SF8/pKC428, E. coli K12 SF8/pKC448, E. coli SF8/pKC462A and E. coli K12 SF8/pKC467. Moreover, of this preferred group, cosmids pKC420, pKC462A, and pKC467, and transformants S. ambofaciens/pKC420, S. ambofaciens/pKC462A, S. ambofaciens/pKC467, E. coli K12 SF8/pKC420, E. coli K12 SF8/pKC462A, and E. coli K12 SF8/pKC467 are most preferred.

The recombinant DNA cloning vectors and transformants of the present invention have broad utility and help fill the need for suitable cloning vehicles for use in Streptomyces and related organisms. Moreover, the ability of the present vectors to confer resistance to antibiotics that are toxic to non-transformed host cells, also provides a functional means for selecting transformants. This is important because of the practical necessity for determining and selecting the particular cells that have acquired vector DNA. Additional DNA segments, that lack functional tests for

their presence, can also be inserted in the present vectors and then transformants containing the non-selectable DNA can be isolated by appropriate antibiotic selection. Such non-selectable DNA segments can be  
5 inserted at any site, except within regions necessary for plasmid function, maintainance, and replication, and include, but are not limited to, genes that specify antibiotic modification enzymes, antibiotic resistance, antibiotic biosynthesis, and regulatory genes of all  
10 types.

The present invention further comprises a novel method for using the aforedefined recombinant DNA cosmid shuttle vectors to construct a genomic DNA library, said method comprising:

- 15 a) ligating a genomic DNA segment into a cosmid shuttle vector, as defined earlier;
- b) packaging said ligated cosmid into bacteriophage lambda particles,
- 20 c) transducing said packaged cosmid into E. coli, and
- d) transforming the recombinant cosmid into a Streptomyces host cell.

More particularly and with reference to  
25 Figure 2, cosmid pKC420 DNA was digested with PvuII restriction enzyme to generate a linear fragment with blunt ends. These blunt ends were then dephosphorylated with bacterial alkaline phosphatase (BAP) to prevent their ligation in subsequent reactions. After extrac-  
30 tion and precipitation, the DNA was digested with BamHI

X-6422A

-18-

restriction enzyme to generate two DNA fragments of unequal length, each fragment consisting of a cos site flanked by a reactive BamHI end and a non-reactive PvuII end. The DNA was extracted, precipitated and then  
5 dissolved in TE buffer for subsequent ligation to the insert DNA fragments. Although the preferred embodiment of the method of this invention employs cosmid pKC420, it is apparent that any one of the following cosmids may be used to construct genomic libraries: pKC427, pKC428,  
10 pKC448, pKC462, pKC462A, or pKC467.

Foreign DNA, such as, for example, Strepto-  
myces felleus DNA (NRRL 2251) was partially digested with a restriction enzyme, such as MboI or Sau3A, to generate a size range (average size of 40 kb) of S.  
15 felleus DNA fragments. These fragments subsequently were treated with BAP to prevent any MboI-generated ends from ligating to each other. Thus, the only allowed ligation was between the MboI ends of the insert DNA and the compatible BamHI ends of the cosmid  
20 DNA. The resultant hybrid DNA molecules served as substrates for in vitro packaging of bacteriophage lambda particles. Due to the size selection mechanism of lambda packaging, only those cosmid-insert-DNA molecules in which the cos sequences are 37 to 52 kb apart were  
25 packaged. Because lambda packaging requires two cos sites, one to initiate and another to terminate packaging and is also size selective, large inserts were positively selected in the transductants. After the desired recombinant DNA procedures were accomplished  
30 within the E. coli host system, the particular recombi-

nant plasmid DNA was isolated and then transformed into a suitable Streptomyces host.

5 The method of the present invention enhances both the efficiency and effectiveness of cosmid cloning given the specific construction of these novel cosmid  
10 vectors. For example, the presence of two or more cos sites in cosmid pKC420 eliminates the need to prepare separately two cosmid arms. Secondly, the presence of blunt-end restriction enzyme sites such as the PvuII and  
15 HpaI sites within the multiple cos site region prevents subsequent cosmid concatemerization between the prepared cosmid arms. Ultimately, given the bifunctionality of the present vectors, the cloned DNA can be shuttled into a Streptomyces host cell for functional analysis of the  
20 cloned DNA. This shuttling capability is particularly advantageous over known cosmid vehicles because Strepto-  
myces cosmid vectors have yet to be exploited.

25 Escherichia coli K12 DH1/pKC420, as a source of cosmid vector pKC420 (NRRL B-15837) and E. coli K12 SF8/pKC462A, as a source of cosmid vector pKC462A (NRRL B-15973), can be cultured in a number of ways using any of several different media. Carbohydrate sources which are preferred in a culture medium include, for example, glucose and glycerol, and nitrogen sources include, for  
30 example, ammonium salts, amino acid mixtures, and peptones. Nutrient inorganic salts also are incorporated and include the customary salts capable of yielding magnesium, sodium, potassium, ammonia, calcium, phosphate, chloride, sulfate, and like ions. As is  
necessary for the growth and development of other

microorganisms, essential trace elements also are added. Such trace elements commonly are supplied as impurities incidental to the addition of other constituents of the medium.

5           E. coli K12 DH1/pKC420 and E. coli K12 SF8/pKC462A were grown under aerobic culture conditions over a relatively wide pH range of about 6.5 to 7.4 at temperatures ranging from about 30° to 42°C. For the  
10           production of the cosmid vectors pKC420 and pKC462A in the greatest quantity, however, it is desirable to start with a culture medium at a pH of about 7.4 and maintain a culture temperature of about 37°C. Culturing the E. coli cells under the aforementioned conditions resulted  
15           in a reservoir of cells from which cosmids pKC420 and pKC462A were isolated by techniques well known in the art.

#### Brief Description of the Figures

20           Fig. 1 shows the restriction site and function map of cosmid pKC420.

            Fig. 2 is a schematic outline of the method of constructing genomic DNA libraries using cosmid vector pKC420 and Streptomyces DNA.

25           Fig. 3 is a restriction site and function map of cosmid pKC427.

            Fig. 4 is a restriction site and function map of cosmid pKC428.

30           Fig. 5 is a restriction site and function map of cosmid pKC448.

            Fig. 6 is a restriction site and function map of cosmid pKC462.

X-6422A

-21-

Fig. 7 is a restriction site and function map of cosmid pKC467.

Fig. 8 is a restriction site and function map of cosmid pKC462A.

5 Fig. 9 is a restriction site and function map of plasmid pOJ108.

Fig. 10 is a restriction site and function map of plasmid pOJ111.

10 Fig. 11 is a restriction site and function map of cosmid cos111.

The following non-limiting examples further illustrate and detail the invention disclosed. Both an explanation of and the actual procedures for constructing the invention are described where appropriate.

15

#### Example 1

#### Culture of E. coli K12 DH1/pKC420 and Isolation of Cosmid pKC420

20

##### A. Culture

5 ml. cultures of E. coli K12 DH1/pKC420 (NRRL B-15837) were grown under selective conditions in TY media (1% tryptone, .5% yeast extract, .5% sodium chloride, pH 7.4) according to conventional microbiological procedures. The cells were spun in a table top centrifuge and the pellet resuspended in 1 ml. of 0.3M sucrose, 25 mM EDTA (ethylenediaminetetracetic acid) and 25 mM Tris-HCl pH 8 (Solution I). After transfer

30

X-6422A

-22-

to an Eppendorf tube the cells were centrifuged for about one minute and the pellet was resuspended in 0.5 ml. of Solution I. About 50  $\mu$ l. of freshly made lysozyme (20 mg./ml. in water) was added and the solution was incubated for 10 minutes at 37°C.

After the addition of 250  $\mu$ l. of freshly made lysis mix (2% sodium dodecyl sulfate and 0.3N NaOH), the cells were immediately and completely vortexed. The cells were then incubated for ten minutes at 50°C., cooled and added to 100  $\mu$ l. of phenol-Sevag (phenol-chloroform-isoamyl alcohol, 25-24-1). After the DNA was centrifuged for two minutes in an Eppendorf centrifuge, the supernatant was decanted and transferred to another tube with 70  $\mu$ l. of unbuffered 3M sodium acetate and isopropanol to precipitate the DNA. This solution was incubated for five minutes at room temperature and then centrifuged for two minutes. The supernatant was gently and completely decanted to remove all the excess liquid.

The DNA precipitate was redissolved in 500  $\mu$ l. of TE (10 mM Tris-HCl pH 8 and 1 mM EDTA) and 10  $\mu$ l. of 100 mM Spermine HCl was added. This mixture was vortexed and then incubated for five minutes at room temperature before a five minute spin in an Eppendorf centrifuge. The supernatant was again completely decanted and discarded and the precipitated DNA was vortexed with 1 ml. of 75% ethanol, 0.3M sodium acetate, and 10 mM magnesium acetate. This solution was incubated for five minutes at room temperature and the DNA collected as above. The pellet was redissolved in 10  $\mu$ l. of TE for subsequent use as a cloning vehicle.

Example 2Construction of Cosmid Shuttle Vector pKC4275 A. BamHI Digestion of Cosmid pKC420

About 5 µg. of cosmid pKC420 were digested in 1X BamHI buffer (150 mM NaCl, 6 mM Tris-HCl pH 7.9, 6 mM MgCl<sub>2</sub> and 1 mM Dithiothreitol) in a total volume of 50 µl. with 20 units (New England Biolab) of BamHI restriction endonuclease\*. The mixture was incubated at 37°C. for about 1 hour and then the reaction was terminated by incubation at 70°C. for 10 minutes. Since cosmid pKC420 has a single BamHI site, digestion is easily monitored by agarose gel electrophoresis. The appearance of a single band of about 10 kb signals complete digestion. The DNA was extracted with phenol and Sevag (chloroform:isoamyl alcohol 24:1), precipitated with ethanol, dried and then resuspended in TE for subsequent ligation.

\* Restriction enzymes and instructions can be obtained from the following sources:

25 New England Bio Labs., Inc., 32 Tozer Road, Beverly, Massachusetts 01915

Bethesda Research Laboratories, Inc., P.O. Box 577,  
30 Gaithersburg, Maryland 20760

Boehringer-Mannheim Biochemicals, P.O. Box 50816,  
Indianapolis, Indiana 46250

35



X-6422A

-24-

### B. BclI Digestion of Plasmid pIJ702

About 5 µg. of plasmid pIJ702 DNA (ATCC 39155) were digested in 1X BclI buffer (75 mM KCl, 6 mM Tris-HCl pH 7.4, 10 mM MgCl<sub>2</sub> and 1 mM Dithiothreitol) in a total volume of 50 µl. with 10 units (New England Biolab) of BclI restriction endonuclease. The mixture was incubated at 50°C. for about an hour and then the reaction was terminated by extraction with phenol and Sevag, precipitated with ethanol, dried and then dissolved in 5 µl. TE. The DNA was electrophoresed on a 0.5% agarose gel until the desired ~1 kb BclI fragment was separated from other fragments. Whatman DEAE cellulose paper was placed in a slit prepared ahead of the desired DNA band and the DNA was electrophoresed onto the DEAE paper. The paper was washed with 1 ml. of TE and the DNA was eluted with 400 µl. of TE adjusted to 1M by the addition of an appropriate volume of NaCl. The eluted DNA was ethanol precipitated and finally dissolved in 5 µl. of TE.

### C. Ligation and Construction of E. coli K12 SF8/pKC427

About 1 µg. each of BamHI-digested cosmid pKC420 DNA and the ~1 kb BclI thiostrepton resistance-conferring fragment were ligated in 20 µl. of 1X ligase buffer (50 mM Tris-HCl pH 7.8, 10 mM MgCl<sub>2</sub>, 20 mM Dithiothreitol and 1 mM ATP) with 400 units of T4 DNA ligase\* for 16 hours at 16°C. The reaction was terminated by incubation at 70°C. for 10 minutes. After

cooling on ice, the resultant ligated DNA was used to transform E. coli K12 SF8 (NRRL B-15835) according to the procedure of Maniatis et al., 1982, Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. The identity of the desired transformants was conventionally confirmed by screening for the loss of the BamHI site and the acquisition of a SalI site. Competent cells were stored in 20% glycerol, instead of dimethyl sulfoxide, at -70°C. The resultant E. coli K12 SF8/pKC427 transformants were conventionally cultured for subsequent production and isolation of cosmid pKC427. A restriction site and function map of cosmid pKC427 is presented in Figure 3 of the accompanying drawings.

\*T4 DNA ligase can be obtained from the same sources as those identified for restriction enzymes.

### Example 3

#### Construction of Cosmid Shuttle Vector pKC428

##### A. EcoRI Digestion of Cosmid pKC427

About 5 µg. of cosmid pKC427 DNA were digested in 1X EcoRI buffer (50 mM NaCl, 100 mM Tris-HCl pH 7.5, 5 mM MgCl<sub>2</sub> and 1 mM Dithiothreitol) in a total volume of 50 µl. with 20 units (New England Biolab) of EcoRI restriction endonuclease. The mixture was incubated at 37°C. for about 1 hour and then the reaction was terminated by incubation at 70°C. for 10 minutes. Since

X-6422A

-26-

cosmid pKC427 contains a unique EcoRI site, an EcoRI digestion generates a single linear fragment.

5 B. EcoRI Digestion of Plasmid pUC4K and Isolation of  
the ~1.5 kb EcoRI Neomycin Resistance-Confering Gene

10 The desired digestion was carried out in substantial accordance with the teaching of Example 3A except that plasmid pUC4K (NRRL B-15836) DNA, rather than cosmid pKC427 DNA, was used. The isolation of the ~1.5 kb EcoRI fragment was carried out in substantial accordance with the teaching of Example 2B.

15 C. Ligation and Construction of E. coli K12 SF8/pKC428

20 The ligation and subsequent transformation procedures were carried out in substantial accordance with the teaching of Example 2C. The identity of the desired transformants was conventionally confirmed by initially selecting for Am<sup>R</sup> phenotype and then replicating those Am<sup>R</sup> colonies to select for neomycin resistant colonies. These colonies were additionally screened for the acquisition of BamHI and SalI restriction sites. A restriction site and function map  
25 of cosmid pKC428 is presented in Figure 4 of the accompanying drawings.

Example 4Construction of Cosmid Shuttle Vector pKC4485 A. BamHI Digestion of Cosmid pKC428 and Subsequent  
Ligation

---

10 Cosmid pKC448 was constructed by deleting a  
BamHI fragment from cosmid pKC428. The desired BamHI  
digestion was carried out in substantial accordance with  
the teaching of Example 2A except that cosmid pKC428 DNA  
was used in place of cosmid pKC420 DNA. The resulting  
fragments were recovered and self-ligated in substantial  
15 accordance with the teaching of Examples 2B and 2C.  
This digestion results in the removal of the neomycin  
resistance conferring gene and generates a unique BamHI  
site flanked by two EcoRI sites. A restriction site and  
function map of cosmid pKC448 is presented in Figure 5  
of the accompanying drawings.

20

Example 5Construction of Cosmid pKC46225 A. HindIII and SalI Digestion of Cosmid pKC448

---

About 10 µg. of cosmid pKC448 were digested in  
100 µl. of buffer (150 mM NaCl, 6 mM Tris-HCl pH 7.9, 6  
mM MgCl<sub>2</sub> and 1 mM Dithiothreitol) with 20 units each of  
30 HindIII and SalI restriction enzymes for 2 hours at

X-6422A

-28-

37°C. The DNA was ethanol precipitated and then re-suspended in 20 µl. of TE.

5 B. Digestion of Plasmid pKC7 DNA and Isolation of the Neomycin Resistance-Conferring Gene

10 The desired digestion and isolation of the ~1.5 kb HindIII-SalI fragment is carried out in substantial accordance with the teaching of Example 5A except that plasmid pKC7 (ATCC 37084) is used in place of cosmid pKC448.

C. Ligation and Construction of E. coli DH1/pKC462

15 The ligation and subsequent transformation procedures were carried out in substantial accordance with the teaching of Example 2C except that E. coli DH1 (NRRL B-15021) was used in place of E. coli K12 SF8. In addition, cosmid pKC462 DNA was used in place of cosmid  
20 pKC427 DNA. The identity of the desired transformants was conventionally confirmed by initially selecting for Am<sup>R</sup> phenotype and then replicating those Am<sup>R</sup> colonies to select for neomycin resistant colonies. A restriction site and function map of cosmid pKC462 is presented in  
25 Figure 6 of the accompanying drawings.

Example 6Construction of *E. coli* K12 SF8/pKC448

5           The desired construction was made, selected,  
and recovered in substantial accordance with the  
teaching of Example 2C except that cosmid pKC448 DNA was  
used in place of cosmid pKC427 DNA. The identified  
transformants were then used for subsequent production  
10 and isolation of cosmid pKC448 according to the teaching  
of Example 1.

Example 7

15   Construction of *Streptomyces ambofaciens*/pKC420  
and *S. ambofaciens*/pKC448

          About 1 µg. each of the DNA from Examples 1  
and 6 and 200 µl. of protoplasts of *Streptomyces*  
20 *ambofaciens*, a strain deposited and made part of the  
permanent stock culture collection of the Northern  
Regional Research Laboratories, Peoria, Illinois, from  
which it is available to the public under the accession  
number NRRL 2420, were mixed with 500 µl. of 55% poly-  
25 ethylene glycol (Sigma) in P medium (Hopwood and Wright,  
1978, Molecular and General Genetics 162:307), vortexed,  
and then aliquots of 25 µl. and 250 µl. were plated onto  
R2YE\* plates with 3 ml. of R2YE top agar. The plates  
were incubated for 18 hours at 30°C. and then overlaid  
30 with 3 ml. of R2YE top agar containing sufficient

apramycin\*\* for a final concentration of 50 µg./ml. The plates were then incubated for an additional 3 days at 30°C. The resultant S. ambofaciens/pKC420 and S. ambofaciens/pKC448 apramycin resistant colonies were isolated according to known procedures, cultured, and then conventionally identified by restriction enzyme and agarose gel electrophoretic analysis of their constitutive cosmids (Maniatis et al., 1982).

10     \* R2YE medium was prepared with the following composition per liter:

15           Sucrose-103 g. 2.5% K<sub>2</sub>SO<sub>4</sub>-10 ml. MgCl<sub>2</sub>-10.1 g.  
          Glucose-10 g. Casamino acids-0.1 g. Agar-22 g.  
          Trace Element Mix-2 ml. 0.5% KH<sub>2</sub>PO<sub>4</sub>-10 ml. 1M  
          CaCl<sub>2</sub>-20 ml. Proline-3 g. 0.25M TES pH 7.2-100 ml.  
          10% Yeast extract-50 ml.

20     \*\* Antibiotic apramycin can be obtained from either Sigma, St. Louis, Missouri or Eli Lilly and Company, Indianapolis, Indiana.

#### Example 8

### 25     The Construction of a Genomic Library

#### A. Preparation of the Vector pKC420 DNA

30           About 50 µg. of cosmid pKC420 DNA were digested in 500 µl. of 1X PvuII (60 mM NaCl, 6 mM Tris-HCl pH 7.5, 6 mM MgCl<sub>2</sub> and 1 mM Dithiothreitol) buffer with 100 units of PvuII restriction enzyme for 3 hours at 37°C. About 50 µl. of 10X BAP buffer (500 mM Tris-HCl pH 8 and 500 mM NaCl) and 2.5 units of BAP

X-6422A

-31-

(International Biotechnologies, Inc., P.O. Box 1565, New Haven, CT 06506) were added and incubated for 1 hour at 70°C. The DNA was extracted with phenol, Sevag and precipitated with ethanol. The DNA was then digested in 500 µl. of 1X BamHI buffer with 90 units of BamHI restriction enzyme for 3 hours at 37°C. The DNA was again extracted with phenol, Sevag, precipitated with ethanol and finally dissolved in 50 µl. of TE.

10 B. Preparation of the Insert DNA

Streptomyces felleus (NRRL 2251) was grown in 250 ml. of Tryptic Soy broth supplemented with 100 µg./ml. of spiramycin\* for 16 hours at 30°C. The cells were harvested by centrifugation (10 minutes at 8,000 rpm) suspended in 10 ml. of lysis mix (300 mM Sucrose, 25 mM Tris-HCl pH 8, and 25 mM EDTA) and brought to a final concentration of 1 mg./ml. with lysozyme and incubated at 37°C. for 10 minutes. Then proteinase K was added to a final concentration of 200 µg./ml. and SDS (sodium dodecyl sulfate) was added to a final concentration of 2%. The mixture was incubated at 70°C. for 10 minutes and then cooled on ice. The mixture was made 1M in potassium acetate and left on ice for 30 minutes. After gently extracting the material with TE saturated phenol, the layers were separated and the aqueous layer was gently extracted with Sevag. Layers were again separated and the nucleic acids in the aqueous layer were precipitated with ethanol. The precipitate was washed with 70% ethanol



and then dissolved in 5 ml. TE. RNase A was added to the DNA solution to a final concentration of 50 µg./ml. This solution was then incubated at 37°C. for 30 minutes; extracted twice with phenol, twice with Sevag and then precipitated with ethanol. The DNA was redissolved in 1 ml. TE (545 µg./ml.) and then sized on a 0.3% agarose gel with λ standards and was found to have an average size of 70 kb.

Next, 50 µg. of Streptomyces felleus chromosomal DNA were incubated with 30 units of MboI in 500 µl. of 1X MboI buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.4, 10 mM MgCl<sub>2</sub>, and 1 mM DTT) at 37°C. for 15 minutes. This particular condition was found, empirically, to give the desired partial fragmentation of chromosomal DNA. The DNA was extracted with phenol, Sevag and then dissolved in 50 µl. of TE.

About 25 µg. of Streptomyces felleus MboI partials were subsequently treated with BAP (1.25 units for the first 1 hour at 70°C., and then an additional 1.25 units for another hour at 70°C.) in 100 µl. of 1X BAP buffer. The DNA was extracted with phenol, Sevag, precipitated with ethanol and then dissolved in 50 µl. of TE. The size of this DNA was estimated on a 0.3% agarose gel and was found to be 30-40 kb.

\* Antibiotic spiramycin can be obtained from Sigma, St. Louis, Missouri.

C. Ligation of the Vector DNA to the Insert DNA

About 125 ng. of the pKC420 arms (prepared in Example 8A) were mixed with 500 ng. of Streptomyces felleus MboI partials (prepared in Example 8B) and ligated with 400 units (New England Biolabs) of T4 DNA ligase in 20  $\mu$ l. of 1X ligase buffer made 1 mM in ATP. Ligation was performed for 16 hours at 16°C. and then terminated by heating for 10 minutes at 70°C.

D. In Vitro Packaging

Packaging was performed by adding about 10  $\mu$ l. of the ligation mixture (~62.5 ng. of hybrid vector DNA) to Biotec packaging kit\* at 30°C. for 1 hour. To this mixture, about 500  $\mu$ l. of 0.1M NaCl, 0.01M Tris-HCl pH 8, and 0.01M MgSO<sub>4</sub> were added. Lastly, 25  $\mu$ l. of chloroform were added to kill any living bacteria.

\* Promega Biotec, 2800 S. Fish Hatchery Road, Madison, WI 53711.

E. Transduction of E. coli K12 SF8

About 200  $\mu$ l. of packaged cosmids (25 ng. of vector DNA) were adsorbed to 500  $\mu$ l. of E. coli strain K12 SF8 grown in Tryptone yeast extract supplemented with 0.2% maltose and 10 mM magnesium sulfate. Adsorption was performed for 10 minutes at 37°C in 10mM Tris pH 8.0 and 10mM MgSO<sub>4</sub>. The cells were grown in 5 ml. of Tryptone yeast extract for three hours at room tem-

X-6422A

-34-

perature and transductants were selected at 30°C. on plates supplemented with 200 µg./ml. of apramycin. Approximately 400 colonies resulted from the plating of 0.1 ml. of transduced cells giving rise to a transducing efficiency of about  $1.2 \times 10^6$  transductants per microgram.

#### F. Transformation into *Streptomyces ambofaciens*

The desired transformation was performed in substantial accordance with the teaching of Example 7. About  $3.4 \times 10^4$  transformants per 1 µg. of *E. coli* grown pKC420 were obtained in this experiment.

#### 15 Example 9

##### Construction of Cosmid pKC467

#### A. XbaI Digestion of Cosmid pKC462

About 25 µg. of cosmid pKC462 are digested with 200 units of XbaI restriction enzyme in 100 µl. of IX XbaI buffer (50 mM NaCl, 100 mM Tris-HCl pH 7.5, 5 mM MgCl<sub>2</sub> and 1 mM Dithiothreitol). The mixture is incubated at 37°C. for about 1 hour and then the reaction is terminated by incubation at 70°C. for 10 minutes. The digested DNA is electrophoresed on a 0.5% agarose gel (International Biotech, Inc.) and then the large fragment is isolated on DEAE paper. The isolated DNA is eluted with 400 µl. TE and 1M NaCl and then ethanol precipitated. The DNA is resuspended in 20 µl. TE for subsequent ligation.

B. Ligation and Construction of *E. coli* K12 SF8/pKC467

About 3  $\mu$ l. ( $\sim$ 1.5  $\mu$ g.) of the isolated DNA are self-ligated and used to transform *E. coli* K12 SF8 in substantial accordance with the teaching of Example 2C. The identity of the desired transformants is conventionally confirmed by initially selecting for  $Am^R$  phenotype and then replicating those  $Am^R$  colonies to select for neomycin resistant colonies. The resultant *E. coli* K12 SF8/pKC467 transformants are conventionally cultured for subsequent production and isolation of cosmid pKC467.

This *Xba*I deletion results in the generation of a low-copy number *Streptomyces* vector. Low-copy number vectors are advantageous in that recipient host strains are not likely to be damaged by possible high-level expression of physiologically active gene products. A restriction site and function map of cosmid pKC467 is presented in Figure 7 of the accompanying drawings.

Example 10

Construction of *Streptomyces ambofaciens*/pKC462 and *S. ambofaciens*/pKC467

About 1  $\mu$ g. of DNA from Example 5 and 200  $\mu$ l. of protoplasts of *Streptomyces ambofaciens* (NRRL 2420) were mixed in substantial accordance with the teaching of Example 7. The identity of the desired transformants

X-6422A

-36-

was conventionally confirmed by initially selecting for Am<sup>R</sup> phenotype and then replicating those Am<sup>R</sup> colonies to select for neomycin resistant colonies. The resultant S. ambofaciens/pKC462 apramycin resistant and neomycin resistant colonies were isolated according to known procedures, cultured, and then conventionally identified by restriction enzyme and agarose gel electrophoretic analysis of their constitutive cosmids (Maniatis et al., 1982).

10           Streptomyces ambofaciens/pKC467 can be constructed as taught above by substituting the pKC467 DNA from Example 9 for the pKC462 DNA.

#### Example 11

15           Culture of E. coli K12 SF8/pKC462A and Isolation of Cosmid pKC462A

20           The culture of E. coli K12 SF8/pKC462A (NRRL B-15973) and subsequent isolation of cosmid pKC462A were carried out in substantial accordance with the teaching of Example 1. The ~0.4 mg of cosmid pKC462A DNA obtained by this procedure was suspended in 1 ml of TE buffer and stored at -20°C. A restriction site and function map of cosmid pKC462A is presented in Figure 8 of the accompanying drawings. Cosmid pKC462A can be easily distinguished from cosmid pKC462 on the basis of size because cosmid pKC462A contains approximately 0.9 bp more DNA than cosmid pKC462.

30

Example 12Construction of Cosmid pKC467A and *E. coli* K12 SF8/pKC467A

5           The construction of this low copy number Streptomyces vector is performed in substantial accordance with the teaching of Example 9A except that cosmid pKC462A DNA is substituted for the pKC462 DNA.

10           *E. coli* K12 SF8 is transformed with cosmid pKC467A DNA in substantial accordance with the teaching of Example 2C. The identity of the desired transformants can be conventionally confirmed by initially selecting for Am<sup>R</sup> phenotype and then replicating those Am<sup>R</sup> colonies to select for neomycin resistant colonies.

15           The resultant *E. coli* K12 SF8/pKC467 transformants are conventionally cultured for subsequent production and isolation of cosmid pKC467A.

Example 13

20           Construction of Streptomyces ambofaciens/pKC462A and *S. ambofaciens*/pKC467A

25           About 1 µg of the DNA from Example 11 and 200 µl of protoplasts of Streptomyces ambofaciens (NRRL 2420) were individually mixed in substantial accordance with the teaching of Example 7. The identity of the desired transformants was conventionally confirmed by

30           initially selecting for Am<sup>R</sup> phenotype and then replicating those Am<sup>R</sup> colonies to select for neomycin resistant colonies.

Streptomyces ambofaciens/pKC467A can be constructed as taught above by substituting the pKC467A DNA from Example 12 for the pKC462A DNA.

5

Example 14Construction of Cosmid cos111A. Construction of Intermediate Plasmid pOJ107

10

About 25 µg of cosmid pKC462A DNA were digested in 0.5 ml of 1X buffer (150 mM NaCl, 6 mM Tris-HCl pH 7.9, 6 mM MgCl<sub>2</sub> and 1 mM Dithiothreitol) with 20 units of PstI restriction enzyme for 3 hours at 37°C. The DNA was ethanol precipitated and collected by centrifugation. After the DNA pellet was resuspended in 100 µl of TE, the PstI-digested DNA was electrophoresed on a 0.7% agarose mini gel and isolated by electrolution, using an IBI electrolution apparatus. The ~2.0 kb PstI fragment was isolated by running the gel at 150V for 30 minutes, followed by ethanol precipitation and resuspension in 50 µl of TE. About 1 µl of this DNA was digested with 20 units BamHI restriction enzyme at 37°C for 1 hour. The BamHI-PstI-digested DNA was then electrophoresed on a 0.7% agarose gel, and the desired ~1.3 kb BamHI-PstI restriction fragment, containing the apramycin resistance gene, was isolated and purified in substantial accordance with the teaching of Example 2B.

Plasmid pUC19 (commercially available from Pharmacia, Inc., 800 Centennial Dr., Piscataway, N.J.

30

08854) was similarly treated as taught above with BamHI and PstI restriction enzymes and the BamHI-PstI cut plasmid was ligated to the ~1.3 kb BamHI-PstI fragment of cosmid pKC462A and transformed into E. coli K12 SF8 in substantial accordance with the teaching of Example 2C.

The desired transformants, E. coli K12 SF8/pOJ107, were identified by restriction enzyme analysis of their plasmid DNA and by their apramycin-resistant phenotype.

#### B. Construction of Intermediate Plasmid pOJ108

About 10 µg of plasmid pOJ107 DNA were digested in 20 µl of 1X EcoRI buffer with 20 units of EcoRI restriction enzyme. After precipitation with ethanol, these fragments were resuspended in 100 µl of NdeI buffer with 3 units NdeI restriction enzyme. The plasmid DNA mixture was incubated at 37°C for 2 hours and then the DNA was ethanol precipitated, collected by centrifugation, and dissolved in 20 µl of TE. Among the fragments generated by this double digestion is the desired EcoRI-NdeI fragment containing the E. coli replicon and both the ampicillin and apramycin resistance-conferring genes.

Partial NdeI restriction enzyme digests were performed on plasmid pHJL210 DNA (NRRL B-15824) by incubating the EcoRI digested pHJL210 DNA at 37°C for 8 minutes with 3 units of NdeI restriction enzyme. After precipitation, these fragments were cut to completion with 20 units of EcoRI restriction enzyme at 37°C for 2



X-6422A

-40-

hours. The DNA was ethanol precipitated and resuspended in 10  $\mu$ l of TE. This double digest generated the desired EcoRI-NdeI fragment containing the SCP2\* replicon and both the neomycin and thiostrepton resistance-  
5 conferring genes. These two plasmid digests were ligated and used to transform E. coli K12 SF8. The resulting transformants, E. coli K12 SF8/pOJ108, were identified by restriction enzyme analysis of their plasmid DNA and by their apramycin-resistant phenotype. A restriction site  
10 and function map of plasmid pOJ108 is presented in Figure 9 of the accompanying drawings.

C. Construction of Intermediate Plasmid pOJ111

15 Ten  $\mu$ g of plasmid pOJ108 DNA was digested in 100  $\mu$ l of 1X PstI buffer with 50 units of PstI restriction enzyme for 2 hours at 37°C. This PstI digestion of plasmid pOJ108 results in an ~1.0 kb deletion, thereby removing a BamHI site and inactivating the neomycin  
20 resistance-conferring gene. Upon transformation of E. coli K12 SF8 with the then ligated plasmid DNA, the resultant transformants were isolated and identified as plasmid pOJ111. A restriction site and function map of plasmid pOJ111 is presented in Figure 10 of the accom-  
25 panying drawings.

D. Preparation of ScaI-HindIII-Digested Plasmid DNA

About 5  $\mu$ g of plasmid pOJ111 DNA were mixed  
30 with 100  $\mu$ l 1X reaction buffer (150 mM NaCl, 6 mM

X-6422A

-41-

Tris-HCl pH 7.5, 6 mM MgCl<sub>2</sub> and 6 mM Dithiothreitol), and 3 µl (~50 units) of ScaI restriction enzyme and the resulting reaction was incubated at 37°C for 2 hours. The DNA was extracted with phenol and Sevag, precipi-  
5 tated with ethanol and resuspended in 20 µl of TE. The DNA was resuspended in HindIII buffer and then cut with 50 units of HindIII restriction enzyme at 37°C for 2 hours. After extraction with phenol and Sevag, the DNA  
10 was precipitated with ethanol and resuspended in 20 µl of TE.

Ten µg of plasmid pKC462A DNA were digested with HindIII and ScaI (increased to 70 units) restriction enzymes as taught above. This double digestion generates the desired HindIII-ScaI fragment containing  
15 the multiple cos sites and part of the ampicillin resistance-conferring gene. After the reaction ~3 µl (~45 units) of XhoI restriction enzyme were added to the reaction, which was then incubated at 37°C for 2 hours. This XhoI digestion serves to reduce the likelihood of  
20 parental plasmids from reappearing. The digested DNA was isolated as taught above.

E. Ligation of Fragments to Construct Cosmid cos111 and Transformation of E. coli K12 SF8

25 Five µl of the ScaI-HindIII restriction fragments of plasmid pOJ111 prepared in Example 14D and 5 µl of the ScaI-HindIII digest of cosmid pKC462A prepared in Example 14D were mixed together, and ligated. This  
30 ligation mix was used to transform E. coli K12 SF8.

X-6422A

-42-

The desired E. coli K12 SF8/cos111 transformants were identified by their apramycin-resistant phenotype and by restriction enzyme analysis of their cosmid DNA. Cosmid DNA was isolated from the transformants in substantial accordance with the procedure of Example 1. A restriction site and function map of cos111 is presented in Figure 11 of the accompanying drawings.

#### Example 15

10

#### Construction of Streptomyces lividans TK23/cos111

About 1 µg of the DNA from Example 14 and 200 µl of protoplasts of Streptomyces lividans TK23 (NRRL 15826) were individually mixed in substantial accordance with the teaching of Example 7. The identity of the desired S. lividans TK23/cos111 transformants was conventionally confirmed by initially selecting for the apramycin-resistant phenotype and then replicating those apramycin-resistant colonies to select for thiostrepton resistant colonies.

20

CLAIMS

1. A recombinant DNA cosmid shuttle vector  
which comprises:

- 5           a) a replicon that is functional in E. coli,  
          b) a replicon that is functional in  
              Streptomyces,  
          c) a DNA segment that contains two or more  
              cos sequences of bacteriophage lambda,  
10           and  
          d) one or more DNA segments that convey  
              resistance to at least one antibiotic  
              when transformed into a sensitive re-  
              strictionless host cell.

15           2. A recombinant DNA cosmid shuttle vector  
as claimed in Claim 1 selected from pKC420, pKC427,  
pKC428, pKC448, pKC462, pKC467, pKC462A, pKC467A and  
cos111.

20           3. A recombinant DNA cosmid shuttle vector as  
claimed in claim 1 selected from pKC420, pKC427, pKC428,  
pKC448, pKC462 and pKC467.

          4. Cosmid shuttle vector pKC420, pKC427,  
pKC428, pKC448, pKC462, or pKC467.

25           5. Cosmid shuttle vector pKC462A, pKC467A, or  
cos111.

          6. A recombinant DNA cosmid shuttle vector  
as claimed in Claim 1 in which the E. coli replicon is  
selected from the replicon-containing fragments of  
plasmids pBR322, pBR324, pBR325 and pBR328.

30           7. A recombinant DNA cosmid shuttle vector  
as claimed in Claim 1 in which the Streptomyces replicon

is selected from the replicon-containing fragments of plasmids SCP2, SCP2\*, SLP1 and pEL103 and pFJ265.

8. A transformed host cell selected from a restrictionless Streptomyces and E. coli, said host cell comprising a recombinant DNA cosmid shuttle vector as claimed in claim 1.

9. A restrictionless Streptomyces or E. coli host cell transformed by a recombinant DNA cosmid shuttle vector as claimed in any one of claims 1 to 6.

10. A host cell as claimed in claim 8 or 9 which is selected from Streptomyces ambofaciens, Streptomyces aureofaciens, Streptomyces cinnamonesis, Streptomyces fradiae, Streptomyces granuloruber, or Streptomyces lividans.

11. Streptomyces ambofaciens/pKC420, Streptomyces ambofaciens/pKC448, Streptomyces ambofaciens/pKC462, Streptomyces fradiae/pKC420, Streptomyces fradiae/pKC448, Streptomyces fradiae/pKC462, Streptomyces lividans/pKC420, Streptomyces lividans/pKC448, Streptomyces lividans/pKC462, Streptomyces lividans/pKC467, E. coli K12 SF8/pKC420, E. coli K12 SF8/pKC448, E. coli DH1/pKC462, Streptomyces ambofaciens/pKC462A, Streptomyces lividans/cos111, E. coli K12 SF8/pKC462A, E. coli K12 SF8/pKC467A or E. coli K12 SF8/cos111.

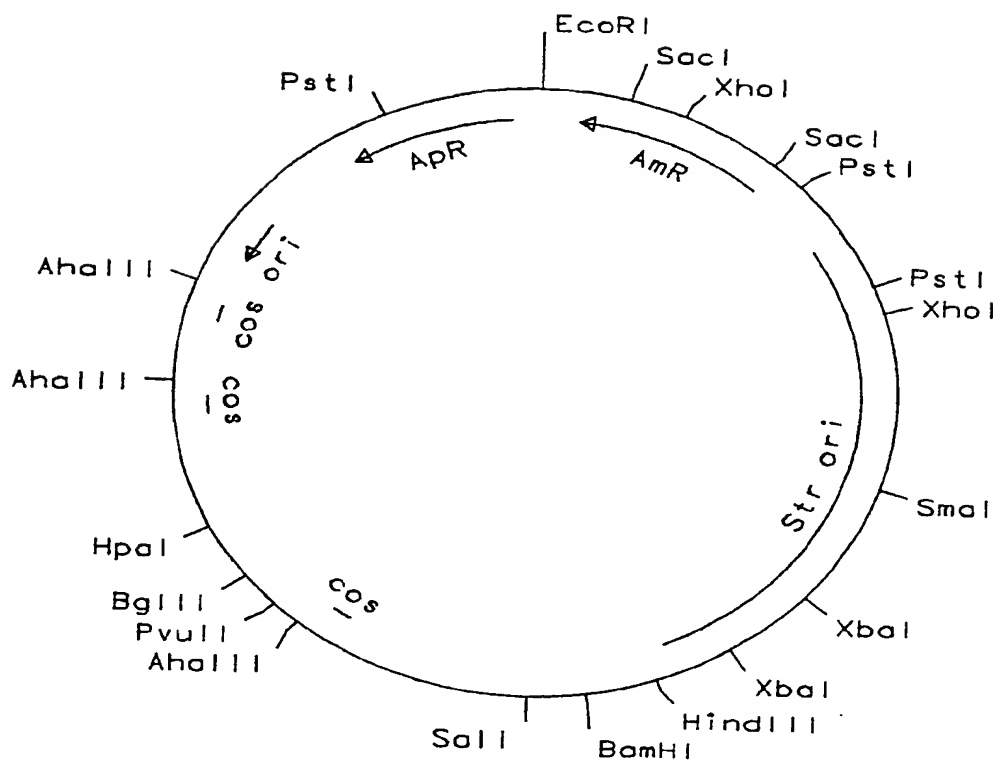
12. A method for constructing a genomic DNA library, which comprises:

- a) ligating a genomic DNA segment into a cosmid shuttle vector as claimed in any one of claims 1 to 7;

5

- b) packaging said ligated cosmid into bacteriophage lambda particles;
- c) transducing said packaged cosmid into E. coli; and
- d) transforming the recombinant cosmid into a Streptomyces host cell.

FIG.1



Restriction Site and Function Map of  
Cosmid pKC420  
(10657bp)

FIG.2

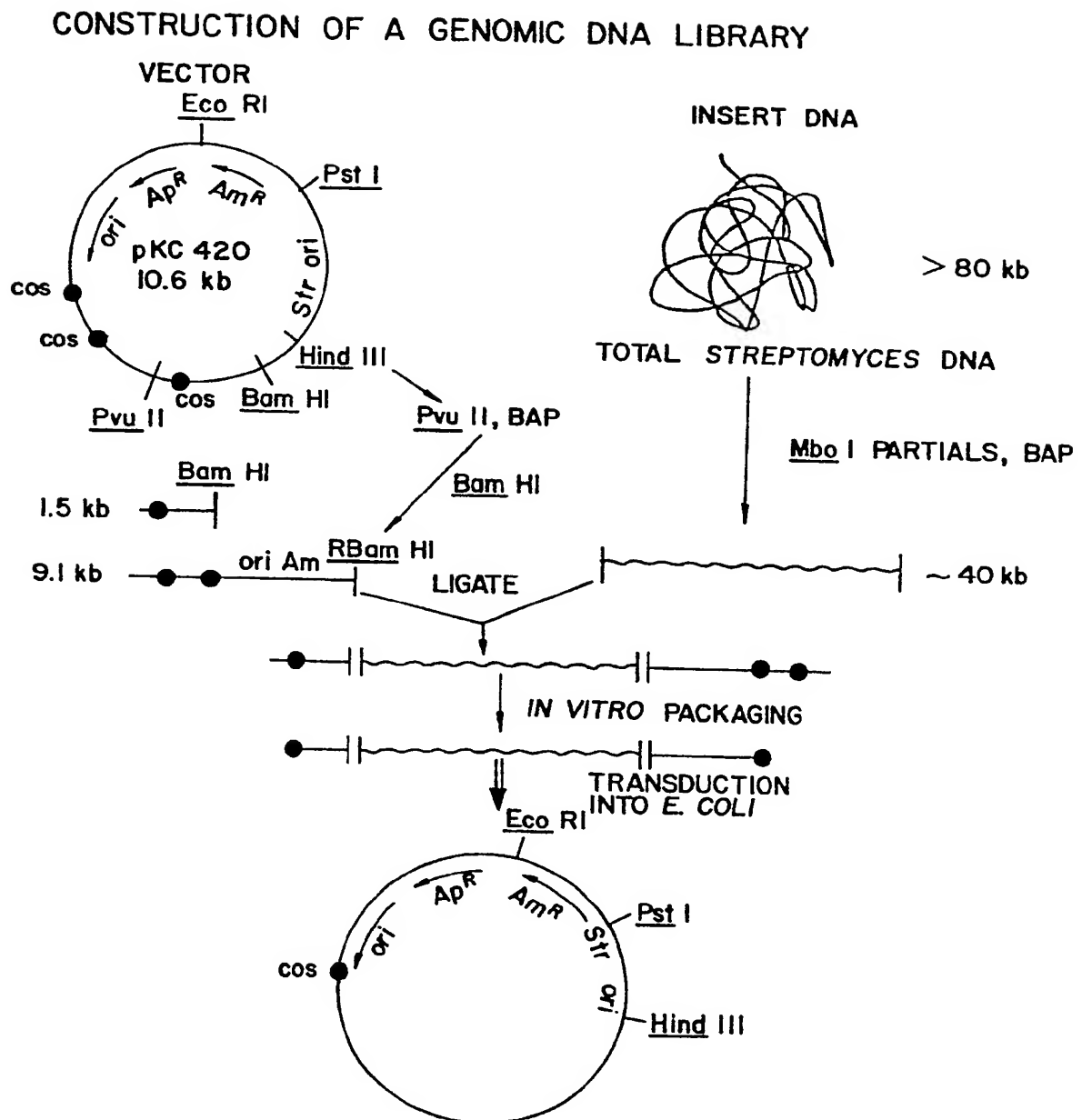
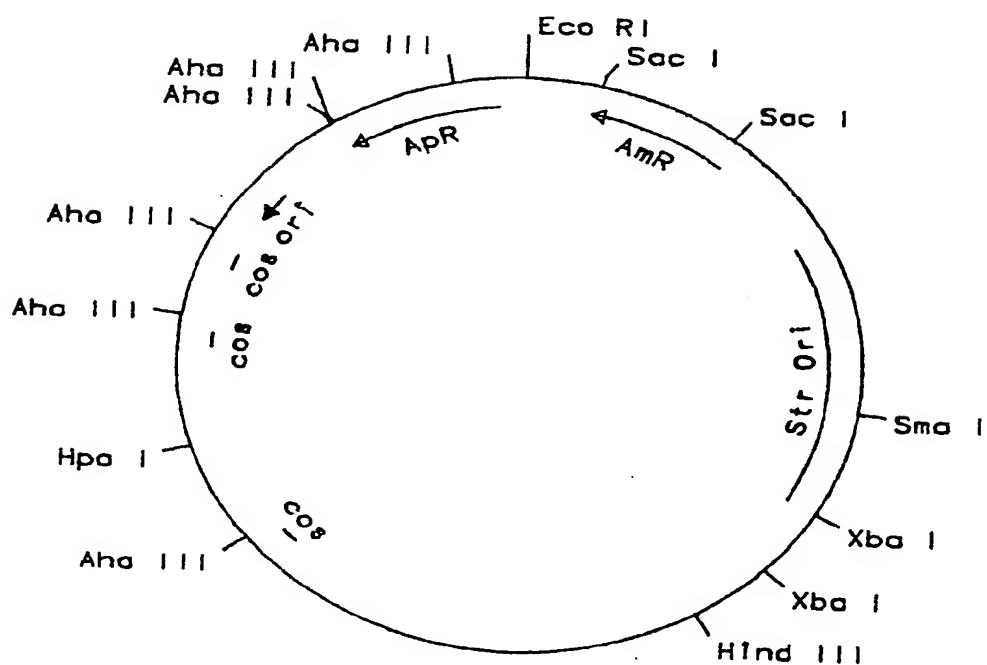


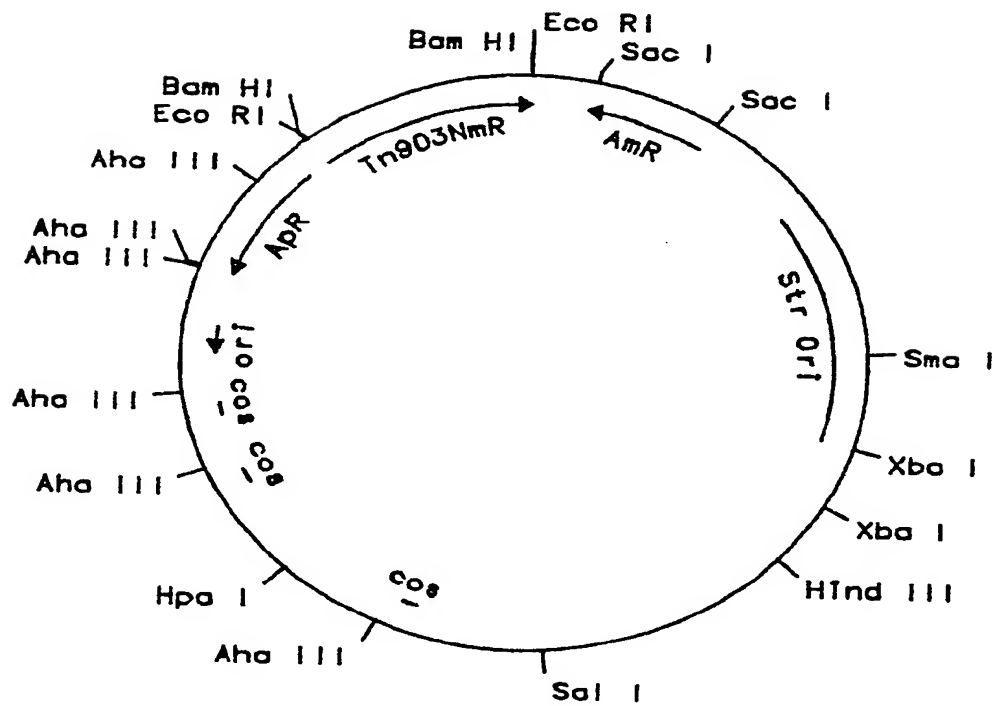


FIG.3



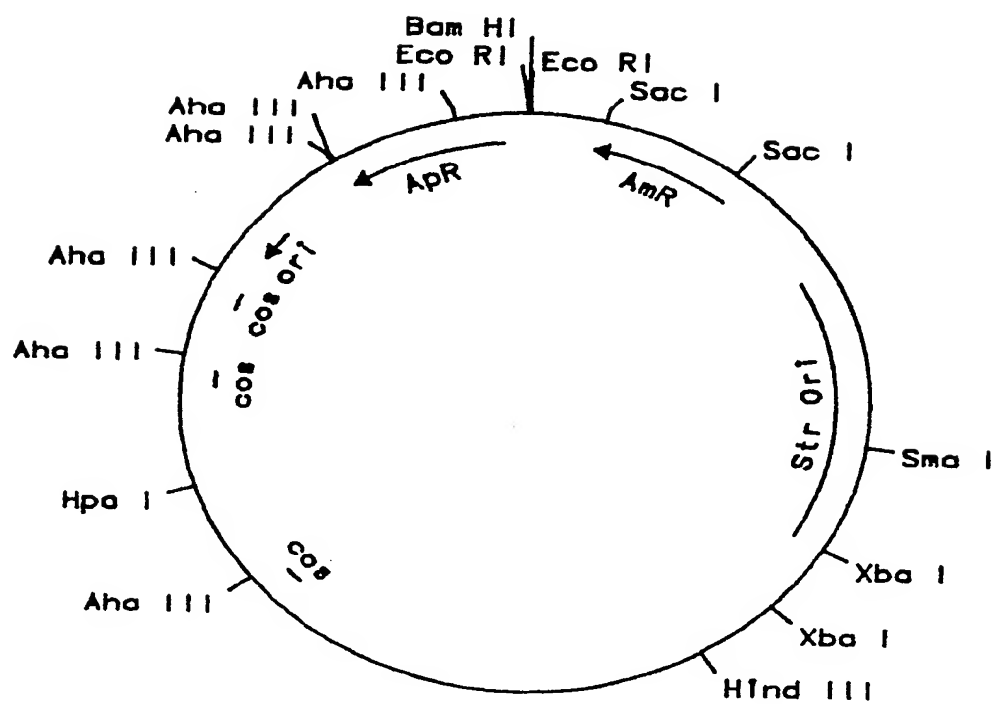
Restriction Site and Function Map of  
Plasmid pKC427  
(11654bp)

FIG.4



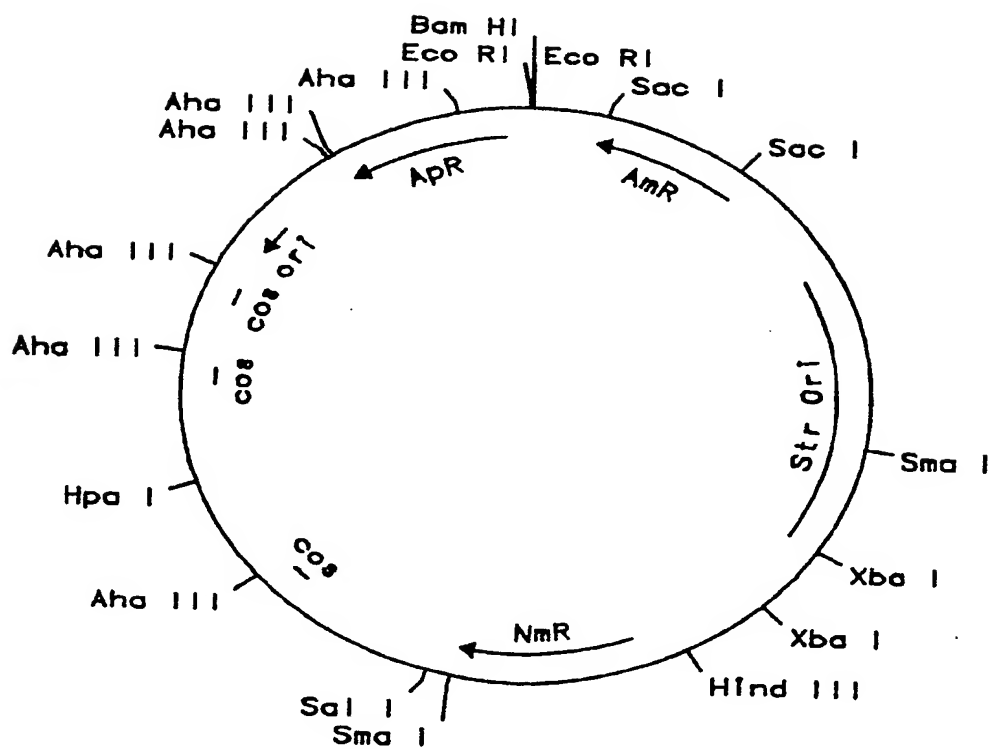
Restriction Site and Function Map of  
Plasmid pKC428  
(13154bp)

FIG.5



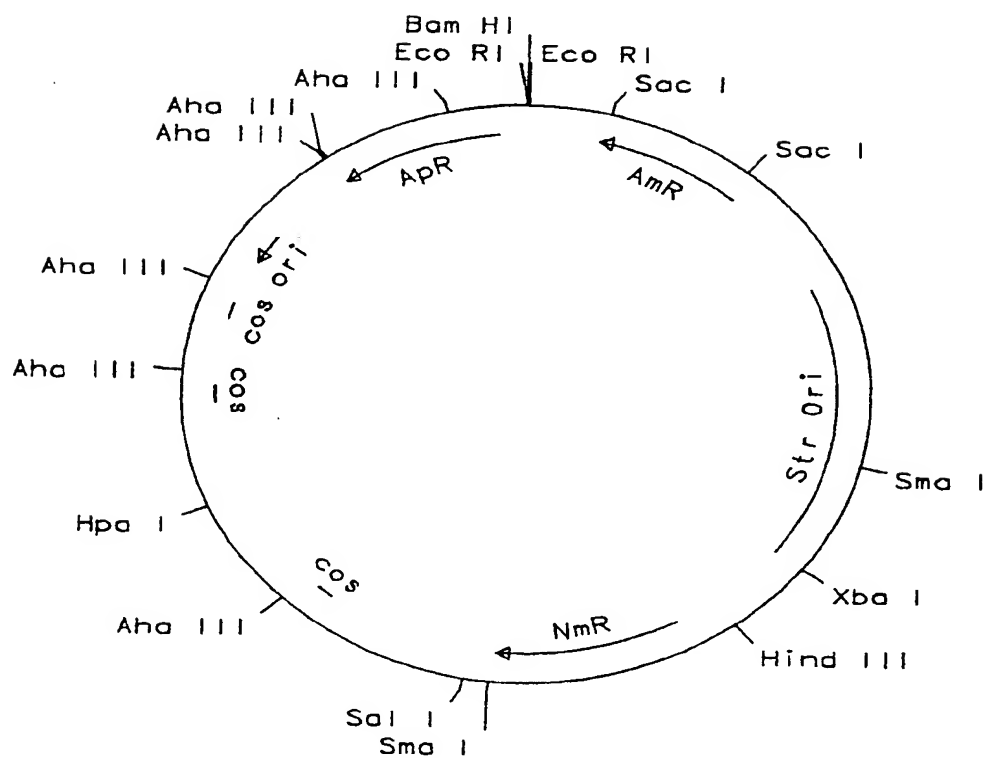
**Restriction Site and Function Map of  
Plasmid pKC448  
(11654bp)**

FIG.6



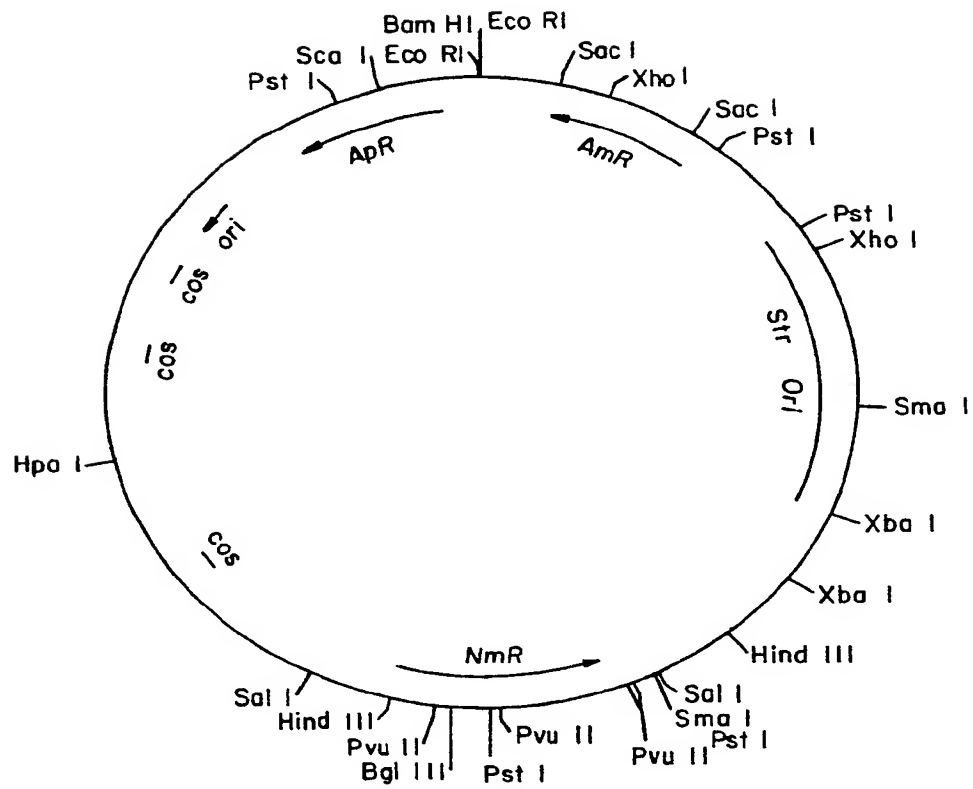
Restriction Site and Function Map of  
Plasmid pKC462  
(11487bp)

FIG.7



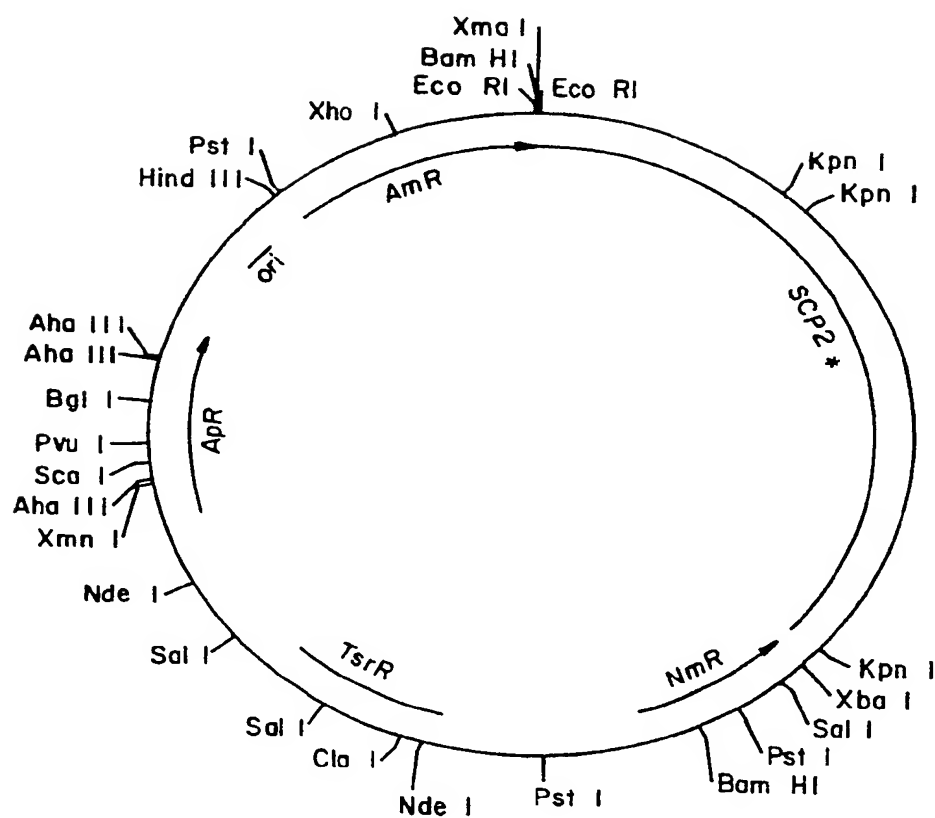
Restriction Site and Function Map of  
Plasmid pKC467  
(11027bp)

FIG. 8



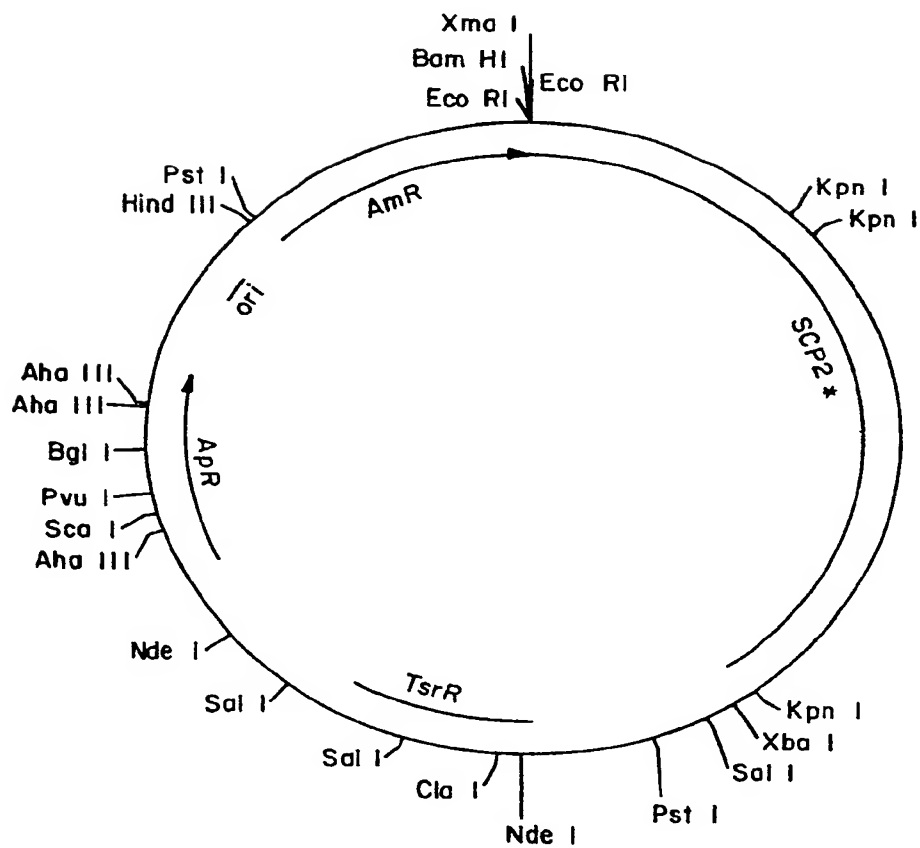
RESTRICTION SITE AND FUNCTION MAP OF PLASMID  
pKC 462A  
(12.4 kb)

FIG. 9



RESTRICTION SITE AND FUNCTION MAP OF PLASMID  
pOJ108  
(12.7 kb)

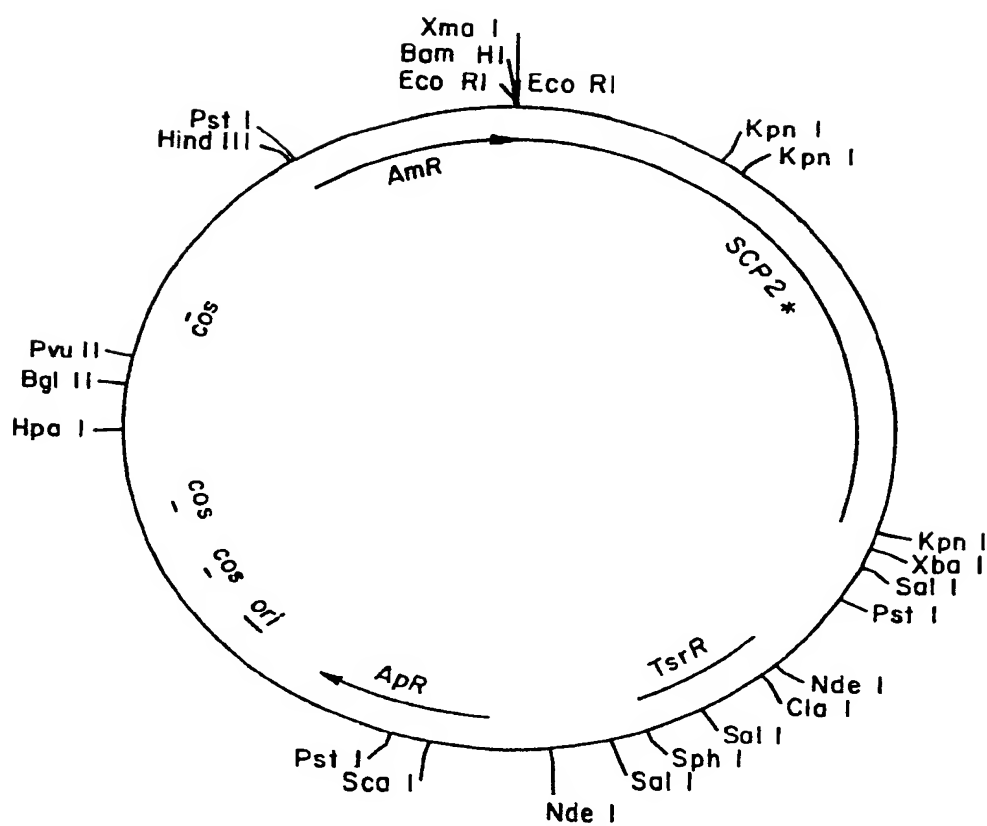
FIG. 10



RESTRICTION SITE AND FUNCTION MAP OF PLASMID  
pOJ III  
(11.7 kb)



FIG. II



RESTRICTION SITE AND FUNCTION MAP OF COSMID  
COS III  
(14.3 kb)



European Patent  
Office

# EUROPEAN SEARCH REPORT

0176921

EP 85306667.8

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
P, A	EP - A2 - O 146 368 (PFIZER LIMITED; PFIZER CORPORATION) * Abstract *	1, 12	C 12 N 15/00 C 12 N 5/00 C 12 N 1/20
A	EP - A1 - O 117 694 (THE UPJOHN COMPANY) * Abstract *	1	//C 12 R 1:19 C 12 R 1:465 C 12 R 1:485 C 12 R 1:54
D, A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 75, no. 9, September 1978, Baltimore, USA J. COLLINS et al. "Cosmids: A type of plasmid gene-cloning vector that is packageable in vitro in bacteriophage $\lambda$ heads" pages 4242-4246 * Totality *	1, 12	-
			TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
			C 12 N
The present search report has been drawn up for all claims			
Place of search VIENNA		Date of completion of the search 02-01-1986	Examiner WOLF
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

EPO Form 1503 (03.82)

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**